

In vitro Synthesis, Glycosylation, and Membrane Insertion of the Four Subunits of Torpedo Acetylcholine Receptor

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Notes:

***In vitro* synthesis, glycosylation, and membrane insertion of the four subunits of *Torpedo* acetylcholine receptor**

(cell-free translation of electroplax mRNA/immunoprecipitation by subunit-specific antisera/transmembrane orientation of acetylcholine receptor subunits/biosynthesis of multisubunit integral membrane proteins)

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ABSTRACT We have characterized the early biosynthetic forms of the *Torpedo* electroplax acetylcholine receptor by using a cell-free protein synthesizing system. We obtained primary translation products of ≈ 38 , 50, 49, and 60 kilodaltons for the α , β , γ , and δ polypeptides, respectively, by using immunoprecipitation with subunit-specific antisera. These chains could each be labeled by the formylated initiator [35 S]Met-tRNA. On cotranslational incubation with pancreatic rough microsomes, glycosylated forms of each subunit were obtained that had molecular weights close to those of their mature authentic counterparts. Extensive trypsinization reduced the glycosylated forms of the receptor subunits to glycosylated membrane-protected fragments of ≈ 35 (α), 37 (β), 45 (γ), and 44 (δ) kilodaltons. In this system, then, each receptor chain spans the membrane at least once. This *in vitro*-synthesized material apparently exhibited neither oligomeric assembly nor α -bungarotoxin binding.

The acetylcholine receptor (AChR) of electric ray and eel is one of the few multisubunit plasma membrane proteins that have been isolated and extensively characterized (for review, see ref. 1). Located in the postsynaptic plasma membranes of the electric organ, the AChR is an oligomer of four polypeptide chains that have masses of 40 (α), 50 (β), 60 (γ), and 65 (δ) kilodaltons (kDa) (2) in the stoichiometry $\alpha_2\beta\gamma\delta$ (3). This purified complex has been shown to form a cation conductance channel in response to carbamoylcholine when reconstituted into planar lipid bilayers (4).

In cultured skeletal myotubes, newly synthesized AChRs require 3 hr to be transported to the plasma membrane (5–7). Although muscle AChR is known to be a complex of five subunits (8), the discrete molecular events that underlie the kinetics of its assembly are not known; the small quantities of muscle receptor thus far available have precluded the preparation of subunit-specific antibodies.

We have studied the early events in AChR biosynthesis by using subunit-specific antisera to characterize the four *Torpedo* receptor polypeptide chains synthesized in a cell-free system. We report here that each subunit is synthesized as an individual polypeptide and that all four chains are independently integrated into dog pancreas microsomes as transmembrane proteins. The mechanism of integration appears to involve a cotranslational process analogous to that described for viral membrane glycoproteins (9–12).

METHODS

Purification of AChR and Preparation of Subunit-Specific Antibodies. AChR was purified in Triton X-100 or Na cholate

from frozen *Torpedo* electroplax tissue by affinity chromatography on cobratoxin-Sepharose (13). Antibodies were prepared against individual subunits eluted from preparative gels in rats as described by Lindstrom *et al.* (14).

Extraction of *Torpedo* Total Cellular RNA. RNA was extracted from frozen pulverized electroplax by a NaDodSO₄/phenol/proteinase K method (15) with an additional LiCl-precipitation step. The homogenate was centrifuged free of glycogen before the first isopropanol precipitation.

Cell-Free Protein Synthesis. Total cellular RNA was translated at a final concentration of 5.0 A₂₆₀ units/ml, for 90 min at 26°C in a micrococcal nuclease-digested wheat germ system containing [35 S]methionine at 0.75 mCi/ml (1 Ci = 3.7×10^{10} becquerels) as described (16), except that the reaction mixtures also contained calf liver tRNA (200 μ g/ml; Boehringer Mannheim) and human placental RNase inhibitor (≈ 16 units/ml) (17). The preparation of canine pancreatic rough microsomes has been detailed elsewhere (18). The translocation activity of these membranes was enhanced with rough endoplasmic reticulum signal recognition protein (18) at 12 equivalents per μ l of membranes in a 200- μ l reaction mixture. Calf liver tRNA was aminoacylated in the presence of [35 S]methionine and formylated as described (19). The preparation was free of [35 S]Met-tRNA^{Met} by the assay of Mihara and Blobel (19). Gel electrophoresis was performed throughout on NaDodSO₄/7.5–15% linear gradient polyacrylamide gels as described (12), and isotopically labeled proteins were visualized by fluorography (20) of the stained dried gels.

Immunoprecipitation. Samples were solubilized in 1% NaDodSO₄ either at 100°C (β , γ , and δ subunits) or 25°C (α subunit) for 5 min, immunoprecipitated, and washed (19) in buffers containing 0.1% Triton X-100/0.2% NaDodSO₄ using protein A-Sepharose 4B as the immunoadsorbent.

Materials. *Torpedo californica* electroplax tissue frozen in liquid N₂ was obtained from Pacific Biomarine (Venice, CA). Nikkol was from Nikko Chemicals, Tokyo, Japan. [35 S]Methionine (1000 Ci/mmol, 1 Ci = 3.7×10^{10} becquerels) and IODOGEN were from New England Nuclear. Cobratoxin and α -bungarotoxin were from Miami Serpentarium Laboratories (Miami, FL). *Escherichia coli* Met-tRNA aminoacyl synthetase and transformylase were gifts from H. Weissbach, and signal recognition protein (18) was the gift of P. Walter.

RESULTS

The antisera used were generated by immunizing rats (14) with denatured AChR subunits that had been cut from preparative NaDodSO₄ gels (Fig. 1A). The actual subunit specificities of the antisera were determined by immunoprecipitation of radioiso-

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Abbreviations: AChR, acetylcholine receptor; Dal, dalton; Con A, concanavalin A.

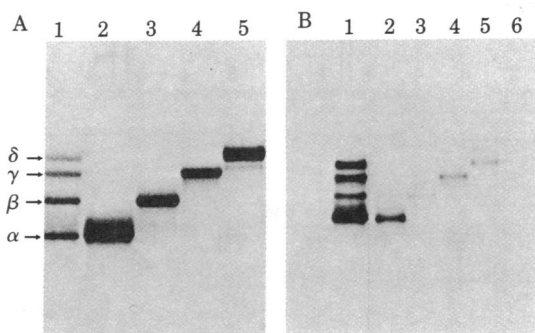


FIG. 1. (A) Coomassie brilliant blue staining pattern of purified AcChoR subunits separated on a NaDodSO₄/7.5–15% linear gradient polyacrylamide gel. Lanes: 1, eluate from cobratoxin affinity column; 2–5, α , β , γ , and δ subunits, respectively, eluted from preparative gels of material shown in lane 1 and rerun. (B) Characterization of anti-AcChoR subunit-specific antisera by immunoprecipitation of radioiodinated authentic AcChoR. Material from lane 1 of A was iodinated in 0.1% Na cholate using IODOGEN (21) to a specific activity of 10^6 cpm/pmol (50% counting efficiency). 9S ¹²⁵I-labeled AcChoR was isolated by sedimentation on 5–20% (wt/wt) linear sucrose gradients. For each immunoprecipitation, ≈ 12 fmol of this material denatured in 1% NaDodSO₄ was incubated with 3 μ l of antiserum (determined to be a slight excess by titration). Samples were reduced with 1 M dithiothreitol and subjected to electrophoresis, and the fixed dried gels were autoradiographed. Lanes: labeled 1, 12 fmol of ¹²⁵I-AcChoR; 2–6, immunoprecipitates with anti- α , β , γ , and δ subunit antisera and non-immune serum, respectively. The incomplete recovery of input material may be due to the lower affinity of protein A for rat IgG and to incomplete elution from the immunoadsorbent during sample preparation.

denated *Torpedo* AcChoR that had been denatured in NaDodSO₄. Under these conditions, the different antisera brought down primarily those subunits to which they had been raised (Fig. 1B).

We demonstrated the *in vitro* synthesis of each of the AcChoR subunits by immunoprecipitation of total translation products with the sera described above. The primary translation products corresponding to each of the four subunits are shown in Figs. 2 and 3, lanes 2. Each of the antisera brought down a distinct polypeptide. In all cases except that of the β subunit the apparent mobility of these AcChoR subunits (synthesized in the absence of rough microsomes) was greater than that of the corresponding authentic chain (compare lanes 1 and 2, Figs. 2 and 3). The β subunit in contrast migrated to the same position as its *in vivo*-synthesized counterpart. The identity of these immunoreactive translation products was confirmed by competition with purified denatured authentic subunits (Figs. 2 and 3, lanes 7). This inhibition of immunoprecipitation did not occur when the unlabeled competing subunit was different from that to which the antiserum was originally raised (not shown). Each subunit constitutes $\approx 0.5\%$ of the total translation products (data not shown).

We found that each of the *in vitro*-synthesized AcChoR subunits required a different sample preparation condition for electrophoresis in order to migrate as a homogeneous species in the gel system used (Figs. 2 and 3). Alteration of these conditions led to the subunits running as multiple species. For example, Mendez *et al.* (22) reported five immunoreactive AcChoR translation products, including a very broad band at ≈ 35 kDa. This smeared band is a conformer of the α subunit, which is converted to the 38-kDa band (Fig. 2A, lane 2) if heating is omitted from the sample preparation (see also Fig. 6.)

In skeletal muscle, "newly synthesized" AcChoRs have been observed in the Golgi apparatus (23). This observation suggests

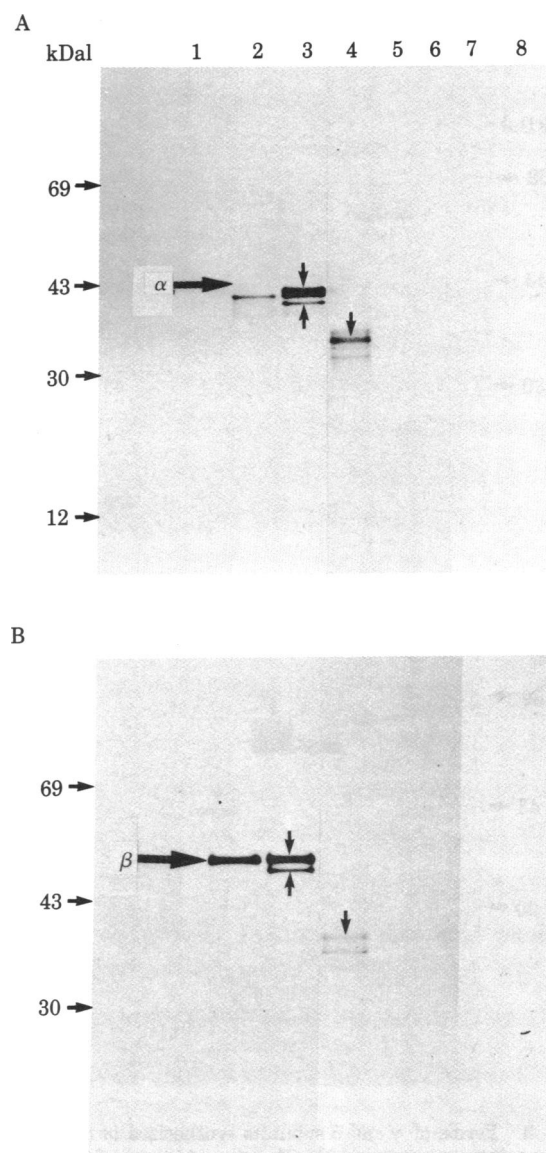


FIG. 2. Forms of α and β AcChoR subunits synthesized *in vitro*. Translations were immunoprecipitated with anti- α (A) or anti- β (B) antisera. Lanes: 1, the M_r of purified unlabeled subunit was marked by aligning the film with the stained gel; 2 and 7, 25- μ l aliquots synthesized in the absence of membranes immunoprecipitated in the absence or presence, respectively, of 1–3 μ g of unlabeled purified subunit; 3 and 8, 50- μ l aliquots synthesized in the presence of membranes immunoprecipitated in the absence or presence, respectively, of unlabeled competing subunit; 4–6, products immunoprecipitated after digestion with trypsin at 300 μ g/ml for 30 min at 26°C in the presence of 5 mM CaCl₂. Digestions were terminated by addition of Trasylol (100 units/ml)/10 mM EDTA followed by NaDodSO₄ solubilization. Lanes 4, a 100- μ l aliquot synthesized in the presence of membranes; lanes 5, as in lane 4 but proteolysis was carried out in the presence of 0.1% Nikkol; lanes 6, a 50- μ l aliquot synthesized in the absence of membranes. In all cases, the samples synthesized in the absence of membranes were incubated posttranslationally for 90 min at 26°C with membranes at the concentration used in the other samples (2.0 A_{280} units/ml). In both cases, samples were prepared for electrophoresis by reduction with 1 M dithiothreitol at 25°C (A) or 100°C (B) for 5 min.

that AcChoRs may be initially synthesized in the rough endoplasmic reticulum membrane system. To examine the polypeptides characteristic of this early stage in AcChoR biosynthesis, we translated *Torpedo* mRNA in the presence of dog pancreas rough microsomal membranes.

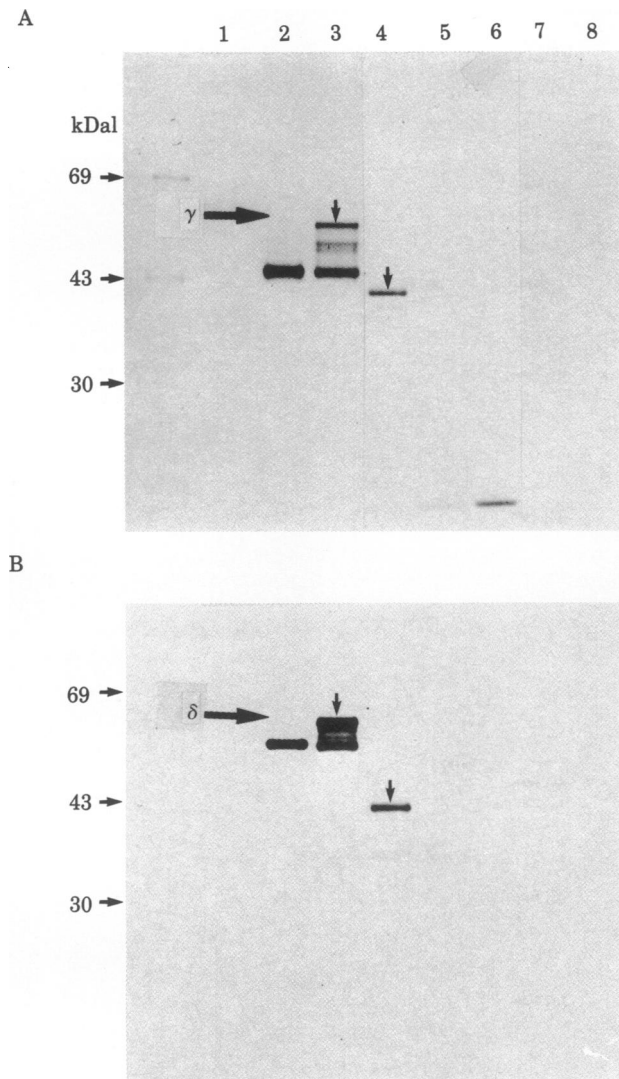


FIG. 3. Forms of γ and δ subunits synthesized *in vitro*. Translations were immunoprecipitated with anti- γ (A) or anti- δ (B) antiserum. Lanes and conditions are exactly as described in the legend to Fig. 2 except that samples were prepared for electrophoresis by reduction for 3 min at 100°C in the presence of 100 mM dithiothreitol and alkylation at 37°C for 45 min with 500 mM iodoacetamide.

The *in vitro*-synthesized AcChoR polypeptides obtained under these conditions are shown in Figs. 2 and 3, lanes 3. The γ and δ subunits exhibit new forms that have greatly increased apparent molecular weights (Fig. 3, lanes 3, arrows) that migrate close to the positions of their corresponding authentic forms (lanes 1). Some minor bands that have apparent mobilities midway between the "precursor" and "authentic" forms can also be observed. In contrast, the α and β subunits undergo little or no increase in their molecular weights, respectively, on addition of membranes (Fig. 2, lanes 3, downward arrows). The shift up for the α subunit, however, is sufficient to bring it close to the position of authentic α subunit. Both the α and β subunits also show forms of lower molecular weight (Fig. 2, lanes 3, upward arrows). All of these membrane-dependent forms can also be immunocompeted (Figs. 2 and 3, lanes 8) by the corresponding authentic subunits, indicating their immunological identity with material synthesized in the absence of membranes.

The synthesis of more slowly migrating forms in the presence of membranes, especially for the γ and δ subunits, appeared

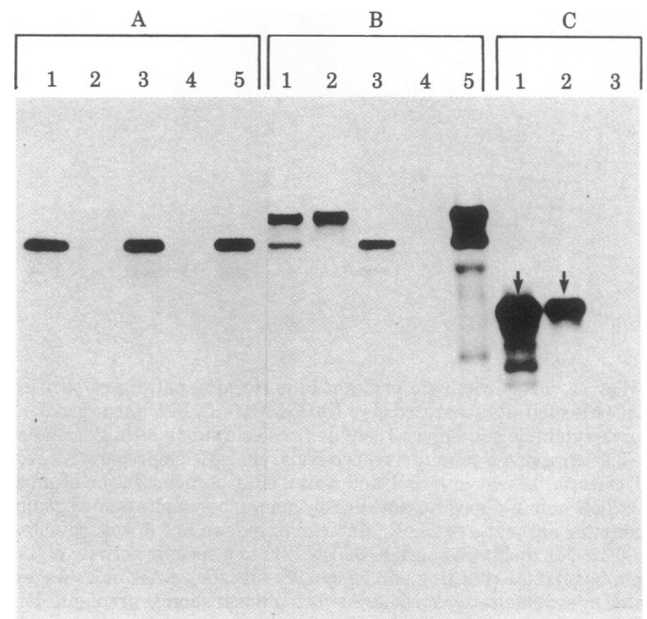


FIG. 4. Fractionation of *in vitro*-synthesized forms of the δ subunit by affinity chromatography on Con A-Sepharose. (A) Products synthesized in the absence of membranes and incubated with microsomes posttranslationally. (B) Products synthesized in the presence of membranes. (C) Products synthesized in the presence of membranes and digested with trypsin. Lanes: 1, aliquots (100 μ l) immunoprecipitated directly without further fractionation; 2–5, aliquots (B, 100 μ l; C, 200 μ l) incubated with Con A-Sepharose in either the absence (lanes 2 and 3) or presence (lanes 4 and 5) of 0.4 M α -methylmannoside. Lanes 2 and 4, material that was adsorbed on lectin-Sepharose; lanes 3 and 5, material that was not retained by Con A. After solubilization in 1% NaDodSO₄ and dilution with 4 vol of buffer containing 1.25% Triton X-100, 5 mM CaCl₂, and 1 mM MgCl₂, the translation products were adsorbed to the Con A-Sepharose by a batch procedure for 4 hr at room temperature. After extensive washing, elution of bound material with 1% NaDodSO₄ and addition of Triton X-100 to 5-fold excess, immunoprecipitation was performed in the normal manner. \downarrow , major tryptic fragment.

similar to that described for the single-subunit transmembrane glycoproteins previously studied in cell-free systems (9, 24, 25). In such cases, these forms were shown to be generated by cotranslational modifications such as glycosylation and signal sequence cleavage (26). The enzymes responsible for these modifications can only be exposed by detergent lysis of the microsomes (18, 27), suggesting that they are located in the luminal space and that the sidedness of these vesicles is uniform. Portions of a nascent chain must therefore be translocated across the bilayer to be so modified. To determine whether these modified AcChoR subunits had untranslocated domains on the cytosolic side of the vesicles, we performed a proteolytic digestion of the *in vitro* translation products before immunoprecipitation.

Extensive trypsinization in all cases converted the subunits synthesized in the presence of membranes to fragments of lower molecular weight (Figs. 2 and 3, lanes 4, downward arrows). The approximate masses of these fragments are 35, 37, 45, and 44 kDa for the α , β , γ , and δ subunits, respectively. In the case of the β subunit, two major fragments are clearly generated (Fig. 2B, lane 4). The origin of the lower band of this doublet is discussed below. In contrast, the material synthesized in the absence of membranes was completely degraded under identical conditions of proteolysis with membranes added posttranslationally (Figs. 2 and 3, lanes 6). This control indicated that the fragments seen in lanes 3 were not due to intrinsic insen-

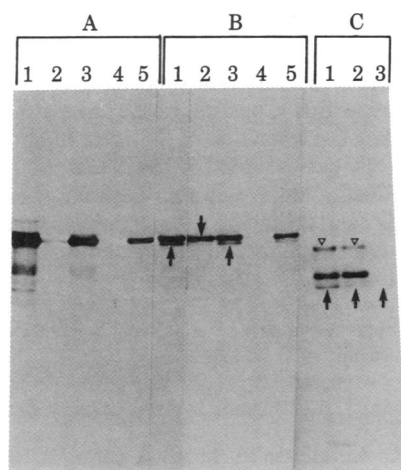


FIG. 5. Fractionation of *in vitro*-synthesized forms of the β -subunit by affinity chromatography on Con A-Sepharose exactly as described in the legend to Fig. 4 for the (δ), subunit. ∇ , Contaminating δ subunit tryptic fragment due to the minor crossreactive antibodies in the anti- β serum, which become significant at the high concentrations of antiserum used in this experiment; \downarrow , glycosylated form of β ; \uparrow , its processed nonglycosylated form or its putative tryptic fragment (B and C, respectively).

sitivity of the subunits to trypsin. Furthermore, when material synthesized in the presence of membranes was similarly digested in the presence of the nonionic detergent Nikkol the fragments also were not present (Figs. 2 and 3, lanes 5). Similar results were obtained by using trypsin plus chymotrypsin (data not shown). These results indicate that all four of the AcChoR subunits have protease-accessible domains at the cytoplasmic surface of the membrane but that substantial portions of each chain are translocated across or embedded in the lipid bilayer (or both) and thereby protected from complete degradation.

All four AcChoR subunits are known to contain carbohydrate (28). To confirm that the mobility shifts seen in the presence of membranes were indeed due to core glycosylation, we fractionated the various *in vitro* products by affinity chromatography on concanavalin A (Con A)-Sepharose 4B before immunoprecipitation. Representative results for the δ and β subunits are shown in Figs. 4 and 5, respectively. In the case of the δ subunit, the two bands immunoprecipitated from the reaction mixtures that contained membranes (Fig. 4B, lane 1) could be fractionated into a major glycosylated form that was adsorbed to the lectin (Fig. 4B, lane 2) and nonglycosylated material that was not retained (Fig. 4B, lane 3). Controls showed that, in the presence of the hapten inhibitor α -methyl mannoside, the glycosylated form was found in the fraction not retained by Con A-Sepharose (Fig. 5B, lane 5). More important, the portion of the δ chain protected by the membrane from trypsin (Fig. 3B, lane 4; Fig. 4C, lane 1) was also adsorbed to the Con A resin (Fig. 4C, lane 2). This indicated that at least part of this membrane-protected domain was completely translocated across the bilayer and exposed to the luminal space. This subunit must therefore span the lipid bilayer at least once. The same behavior was observed for the slower migrating forms of the α and γ subunits and their tryptic fragments (not shown).

In the case of the β subunit, the nonglycosylated and glycosylated forms comigrated in this gel system (Fig. 5A and B, lanes 5 and 1, respectively) but could be resolved by Con A fractionation (Fig. 5B, lanes 1, 2, and 3). Interestingly, the more rapidly migrating membrane-dependent form of the β subunit (compare Fig. 2B, lane 3) appeared primarily in the fraction not retained by the lectin (Fig. 5B, lane 3). This form of the β sub-

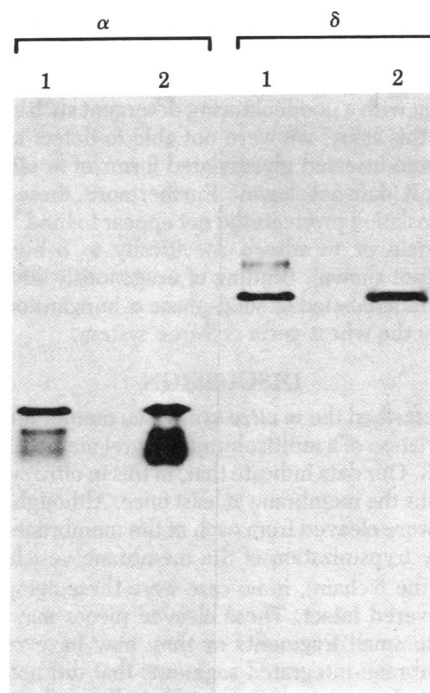


FIG. 6. Labeling of *in vitro* synthesized α and δ subunits with *N*-formyl[^{35}S]methionine. Translations contained RNA at 12 A_{260} units/ml and either [^{35}S]methionine (0.5 mCi/ml; lanes 1) or calf liver tRNA (0.6 mg/ml) containing 6.2×10^7 cpm of ^{35}S as *N*-formyl[^{35}S]Met-tRNA $_{\text{Met}}$ (lanes 2). Translations in lane 1 also contained unlabeled tRNA (0.6 mg/ml) and those in lane 2 contained 2 mM unlabeled methionine. Lanes 1 represent immunoprecipitates derived from 10- μl translation aliquots, and lanes 2 represent products obtained by sequential immunoprecipitation of a single 500- μl translation. All samples were reduced with 1 M dithiothreitol for 3 min at 100°C. As a result, the smeared conformer of the α subunit, which arises as a consequence of heating, and a higher molecular weight form of the δ subunit (lane 1), which arises when the alkylation step is omitted, can be seen.

unit most likely represents material in which the signal sequence has been cleaved and which has been incompletely glycosylated; it would therefore give rise to the smaller of the two tryptic fragments seen in Fig. 2B (lane 4) which, as expected, is also incompletely adsorbed on Con A (Fig. 5C, lanes 2 and 3 arrows). A similar result is obtained for the faster migrating form of the α subunit seen in Fig. 2A (lane 3), although the corresponding tryptic fragment is not as prominent (data not shown). These latter results suggest that some of the chains have undergone membrane integration and processing but not glycosylation (29).

Since there is no precedent for a eukaryotic multisubunit integral plasma membrane protein studied in a cell-free system, we wanted to rule out the possibility that the apparently separate translation products were derived by rapid cleavage of a "polyprotein" precursor. Such precursors have in fact been observed for the transmembrane glycoproteins of some enveloped animal viruses (11). To do this, we asked whether each subunit has a separate ribosomal initiation site by labeling the translation products with formylated initiator methionyl-tRNA. The results for the α and δ subunits (Fig. 6), indicate that both chains are labeled by *N*-formyl[^{35}S]Met-tRNA $_{\text{Met}}$. Similar results were obtained for the β and γ subunits (data not shown). As all eukaryotic mRNAs thus far studied have a single 5'-terminal initiation codon (for review, see ref. 30), this demonstration of separate initiators implies the existence of physically separate mRNAs for each subunit.

We assayed for the assembly of *in vitro*-synthesized AcChoR by asking whether any one of our antisera brought down all four subunits when the immunoprecipitation was performed after solubilization with a nondenaturing detergent such as Triton X-100. Using this assay, we were not able to detect assembly of the membrane-inserted glycosylated forms of *in vitro*-synthesized AcChoR (data not shown). Furthermore, these Triton-solubilized translation products did not appear to bind ¹²⁵I-labeled α -bungarotoxin or to adsorb specifically to α -bungarotoxin-Sepharose (not shown). Binding of exogenously added mature AcChoR to radiolabeled or solid-phase α -bungarotoxin was not inhibited by the wheat germ cell-free system.

DISCUSSION

We have described the *in vitro* synthesis, membrane insertion, and glycosylation of a multisubunit integral membrane protein, the AcChoR. Our data indicate that, in this *in vitro* system, each subunit spans the membrane at least once. Although substantial fragments were cleaved from each of the membrane-integrated subunits by trypsinization of the membrane vesicles (e.g., 20 kDa from the δ chain), in no case were these cytoplasmic domains recovered intact. These cleaved pieces may have been degraded to small fragments or they may have remained as larger membrane-integrated segments that did not react with our antisera. In any case, our results indicate that the greater part of each subunit is protected by the membrane from proteolysis and contains the core oligosaccharide.

By analogy (26), each of these membrane-protected tryptic fragments may contain the NH₂-terminus of the subunit from which it was derived. In the case of the α chain, this has already been confirmed in *Torpedo* plasma membranes (31). Interestingly, for the α and δ subunits, the tryptic fragments we have obtained *in vitro* are similar in size to those generated by proteolysis of alkaline-extracted *Torpedo* plasma membrane vesicles (31). Such fragments have not yet been defined in that system for the β and γ chains.

Our observation that membrane integration and core glycosylation of the four AcChoR subunits requires a cotranslational incubation with rough microsomes suggests that these polypeptides have signal sequences (26) and are synthesized on membrane-bound ribosomes *in vivo* (12). At present we cannot directly compare the early biosynthetic forms of AcChoR obtained *in vitro* and *in vivo*, as there are as yet no culture systems available for *Torpedo* electroplax cells. However, as established in previous cases (9, 10, 24), the cell-free system we have used should have accurately reproduced the events that occur in the living cell.

In support of this is our observation that the major glycosylated forms synthesized in the presence of membranes showed molecular weights similar to those of their authentic counterparts. Exact comigration is not expected, however, as the subunits probably undergo further carbohydrate modifications in the Golgi apparatus. Minor species were also obtained, and we have confirmed by digestion with endo- β -N-acetylglucosaminidase H that these represent partially or nonglycosylated membrane-inserted forms (data not shown).

If, then, each subunit has been correctly inserted into the membrane *in vitro*, why is oligomeric assembly of these chains not observed? One possibility is that the mechanism of assembly may involve aggregation of the subunits by lateral diffusion in the plane of the membrane. As the rough microsomes used *in vitro* are quite small (diameter \leq 3000 Å), the probability of getting one polysome for each subunit on the same vesicle would be very low (given that each chain is only 0.5% of total

protein synthesis) and, therefore, no assembly would be observable. Another possibility is that assembly of the AcChoR requires transport to a different subcellular compartment such as the Golgi apparatus. Consistent with this mechanism, in cultured myotubes the formation of the toxin binding site occurs postsynthetically over a period of 15–20 min. (unpublished observations). Finally, the correlation between the lack of oligomeric assembly and the lack of α -bungarotoxin binding suggests that the α subunit may require interactions with other chains to form the toxin binding site.

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